JC03 Rec'd PCT/PTO 15 JAN 2002

Form PTO-1390 U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE (Rev. 1-98) TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		Attorney's Docket Number 18744-0004 U.S. Application No. (if known, see 37 CFR 1.5) 10/031506
International Application No. PCT/EP00/06832	International Filing Date July 17, 2000	Priority Date Claimed July 15, 1999

Title of Invention
PRODUCTION AND USE OF LUMINESCENT MICROPARTICLES AND NANOPARTICLES

Applicant(s) for DO/EO/US
KLIMANT, Ingo

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), and (9) and (21) indicated below.
4. ☐ The US has been elected by the expiration of 19th month from the earliest claimed priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☒ is attached herewith (required only if not transmitted by the International Bureau).
   b. ☐ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ An English language translation of the International Application into English (35 U.S.C. 371(c)(2))
   a. ☒ is attached herewith.
   b. ☐ has been previously submitted under 35 US.C. 154(d)(4).
   c. ☐ translation not required as the application was filed in English.
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☒ are attached herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
8. ☒ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 20. below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☐ A FIRST preliminary amendment.
14. ☐ A SECOND or SUBSEQUENT preliminary amendment.
15. ☐ A substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 – 1.825.
18. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. ☐ Other items or information:

ATTACH CUSTOMER BAR CODE LABEL BELOW

29052
PATENT & TRADEMARK OFFICE

AO 671214.1

531 Rec'd PCT/PTO 15 JAN 2002

U.S. Application No. (if known, see 37 CFR 1.5) 10/031506	International Application No. PCT/EP00/06832	Attorney's Docket Number 18744-0004

21. ☒ The following fees are submitted: — CALCULATIONS PTO USE ONLY

BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)):

Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO.. **$1040.00**

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$890.00**

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$740.00**

International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$710.00**

International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**

ENTER APPROPRIATE BASIC FEE AMOUNT =				$ 890.00	
Surcharge of $130.00 for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				$	
Claims	Number Filed	Number Extra	Rate		
Total claims	27 - 20 =	7	x **18.00**	$ 126.00	
Independent Claims	1 - 3 =	0	x **84.00**	$ 0.00	
Multiple Dependent Claims (if applicable)			+ **280.00**	$ 0.00	
TOTAL OF ABOVE CALCULATIONS =				$1,016.00	
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by ½. -				$	
SUBTOTAL =				$1,016.00	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				$	
TOTAL NATIONAL FEE =				$1,016.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$ 0.00	
TOTAL FEES ENCLOSED =				$1,016.00	
				Amount to be refunded:	$
				charged:	$

a. ☒ A check in the amount of **$1,016.00** to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. 19-5029 in the amount of $________ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment, to Deposit Account No. 19-5029. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

William L. Warren, Reg. No. 36,714

SEND ALL CORRESPONDENCE TO:
William L. Warren, Esq.
SUTHERLAND ASBILL & BRENNAN, LLP
999 Peachtree Street, N.E.
Atlanta, Georgia 30309
Telephone: 404-853-8000

AO 671214.1

PATENTS

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	Art Unit: **Unassigned**
Ingo Klimant	Examiner: **Unassigned**
Serial No. **Filed Herewith**	
Filed: **January 15, 2002**	
For: **Production and Use of Luminescent Microparticles and Nanoparticles**	
International Application: **PCT/EP00/06832**	
International Filing Date: **July 17, 2000**	
Priority Date: **July 15, 1999**	

PRELIMINARY AMENDMENT

Commissioner for Patents
Washington, DC 20231

Sir:

Please consider the following amendments and remarks prior to calculating the filing fee and the first examination of this Application.

AMENDMENTS

In the Specification

In claim 3, line 1, please delete "claim 1 or 2" and replace therefor --claim 1--.

In claim 5, line 1, please delete "either of claims 3- 4" and replace therefor --claim 3--.

In claim 6, line 1, please delete "claim 1 or 2" and replace therefor --claim 1--.

Express Mail Label No. EL690570129US
January 15, 2002

In claim 7, line 1, please delete "claim 1 or 2" and replace therefor --claim 1--.

In claim 8, line 1, please delete "any of claims 1-7" and replace therefor --claim 1--.

In claim 11, line 1, please delete "any of claims 1-7" and replace therefor --claim 1--.

In claim 13, line 1, please delete "claim 11 or 12" and replace therefor --claim 11--.

In claim 14, line 1, please delete "any of claims 1-13" and replace therefor --claim 1--.

In claim 16, line 1, please delete "any of claims 8-10" and replace therefor --claim 8--.

In claim 18, line 1, please delete "claim 16 or 17" and replace therefor --claim 16--.

In claim 19, line 1, please delete "any of claims 8-10" and replace therefor --claim 8--.

In claim 20, line 1, please delete "any of claims 8-10" and replace therefor --claim 8--.

In claim 22, line 1, please delete "any of claims 11-13" and replace therefor --claim 11--.

In claim 23, line 1, please delete "any of claims 1-14" and replace therefor --claim 1--.

In claim 24, line 1, please delete "any of claims 1-14" and replace therefor --claim 1--.

In claim 26, line 1, please delete "any of claims 1-14" and replace therefor --claim 1--.

In claim 27, line 1, please delete "any of claims 1-15" and replace therefor --claim 1--.

REMARKS

No new matter is contained in the amendment.

The Examiner is encouraged to call the undersigned attorney if doing so will facilitate prosecution of the application. No additional fees are believed due, however, the Commissioner

is hereby authorized to charge any fees due or credit any overpayment to Deposit Account 19-5029.

Respectfully submitted,

Dated: January 15, 2002

William L. Warren
Reg. No. 36,714

SUTHERLAND ASBILL & BRENNAN LLP
999 Peachtree Street, NE
Atlanta, Georgia 30309-3996
(404) 853-8000
Our Docket: 18744-0004

CLEAN SET OF AMENDED CLAIMS PENDING ENTRY OF PRELIMINARY AMENDMENT

1. A luminescent micro- or nanoparticle, characterized in that it contains luminescent substances having long luminescence decay times and said luminescent substances are essentially shielded from ambient chemical, biochemical and gaseous parameters

2. The particle as claimed in claim 1, characterized in that one or more luminescence properties of said luminescent substances, which are in particular selected from the group consisting of quantum yield, spectral characteristics, luminescence decay time and anisotropy, are essentially independent of the particular environment.

3. The particle as claimed in claim 1, characterized in that the luminescent substances are metal/ligand complexes of ruthenium(II), osmium(II) rhenium(I), iridium(III) platinum(II) and palladium(II) as central atom.

4. The particle as claimed in claim 3, characterized in that the luminescent substances are complexes with 2- or 3-dentate polypyridyl ligands such as 2,2'-bipyridine, bipyrazine, phenanthroline, terpyridyl or derivatives thereof as ligands.

5. The particle as claimed in claim 3, characterized in that the luminescent compounds are the tris complexes of ruthenium(II) with 2,2'-bipyridyl, 1,10-

phenanthroline, 4,4-diphenyl-2,2'-bipyridyl and 4,7-diphenyl-1,10-phenanthroline as ligands.

6. The particle as claimed in claim 1, characterized in that the luminescent substances are carbonyl complexes of Re(I) with additional diimine ligands such as derivatives of 2,2'-bipyridyl and 1,10-phenanthroline.

7. The particle as claimed in claim 1, characterized in that the luminescent compounds are porphyrin complexes of Pt(II) and Pd(II) as central atoms.

8. The particle as claimed in claim 1, characterized in that it contains an organic polymer which distinguishes itself by low absorption of water or/and minimum gas permeability.

9. The particle as claimed in claim 8, characterized in that it contains an organic polymer from the group consisting of polyacrylonitrile, poly(meth)acrylic copolymers, polyvinyl chlorides or polyvinylidene chlorides and copolymers thereof.

10. The particle as claimed in claim 9, characterized in that it contains polyacrylonitrile or polyacrylonitrile copolymers, in particular copolymers with acrylic acid, acrylic amines or/and acrylic esters.

11. The particle as claimed in claim 1, characterized in that it contains a glass which is essentially free of micropores.

12. The particle as claimed in claim 11, characterized in that it contains a glass which has been produced according to a sol/gel process.

13. The particle as claimed in claim 11, characterized in that it contains a sol/gel glass which has been prepared from silicon, titanium, zirconium or/and tin tetraalcoholates.

14. The particle as claimed in claim 1, characterized in that its surface has been modified by reactive groups such as amino, epoxy, hydroxyl, thiol or/and carboxyl groups which make possible the covalent coupling of luminescent indicators or/and biomolecules.

15. The particle as claimed in claim 14, characterized in that it contains luminescent indicators or/and biomolecules covalently coupled to its surface.

16. A method for preparing luminescent micro- and nanoparticles as claimed in claim 8, wherein the particles are precipitated from a polymer solution in which the luminescent compound is present in soluble form by adding a liquid dropwise, with the liquid being miscible with the polymer solvent but causing a reduction in the solubility of the polymer.

17. The method as claimed in claim 15, wherein the particles are precipitated from a solution comprising dimethylformamide and polyacrylonitrile or polyacrylonitrile copolymer, in which the

luminescent compound is present in soluble form, by adding water or an aqueous solution dropwise.

18. The method as claimed in claim 16, wherein the particle diameter is adjusted by varying the polymer content of the solution.

19. A method for preparing luminescent micro- and nanoparticles as claimed in claim 8, wherein the luminescent compound is incorporated by diffusion from a solvent (mixture) into already prefabricated particles.

20. A method for preparing luminescent micro- and nanoparticles as claimed in claim 8, wherein the particles are formed by spraying a polymer solution in which the luminescent compound is present in soluble form and evaporation of the solvent.

21. The method as claimed in claim 20, wherein the particle diameter is adjusted by varying the polymer content of the spray solution.

22. A method for preparing luminescent microparticles as claimed in claim 11, wherein the luminescent compound is incorporated into compressed monolithic sol/gel glasses which are subsequently ground and fractionated according to size.

23. The use of the luminescent micro- and nanoparticles as claimed in claim 1 for labeling and luminometric detection of biomolecules from the group consisting of toxins, hormones, hormone receptors, peptides, proteins, lectins, oligonucleotides, nucleic acids, antibodies, antigens, viruses and bacteria.

24. The use of the luminescent micro- and nanoparticles as claimed in claim 1 as reference standards of fluorescence intensity signals in fluorimetric assays.

25. The use as claimed in claim 23, wherein addition of the standard to the sample converts the intensity information into a phase signal or/and a time-dependent parameter.

26. The use of the luminescent micro- and nanoparticles as claimed in claim 1 for referencing the luminescence intensity signal of optical luminescence sensors, wherein the particles are immobilized to a solid phase together with a luminescent indicator.

27. A method for luminometric determination of a biochemical or chemical parameter using two different luminescent dyes which have different decay times and the time or phase characteristics of the resulting luminescent response are used for generating a reference parameter for determination of said parameter, with the first luminescent dye corresponding to said parameter at least with respect to luminescence intensity and the second one not corresponding to said parameter at least with respect to luminescence intensity and luminescence decay time,
characterized in that
the second luminescent dye is used in the form of particles as claimed in claim 1.

10/031506
531 Rec'd PCT/PTC 15 JAN 2002

Preparation and use of luminescent micro- and nanoparticles

Description

The invention relates to the composition, preparation and use of luminescent micro- and nanoparticles with long-lived luminescence. Said particles may be used either as internal standards for referencing fluorescence or phosphorescence signals (luminescence signals) or as markers for labeling and detecting biomolecules. Long-lived luminescent dyes are incorporated in an inert form into solid materials, i.e. shielded from the influence of chemical and biological substances in gaseous and aqueous samples. In this incorporated form, the photophysical properties of the dyes (spectral characteristics, luminescence decay time and luminescence anisotropy) remain unaffected by changing sample parameters.

The incorporating matrix selected is in particular compact inorganic materials or organic polymers which, due to their structure, exclude the uptake of biomolecules, small neutral molecules and also ionic substances. In particular, the interfering influence of molecular oxygen, an efficient fluorescence or phosphorescence quencher, on luminescence measurements is in this way eliminated or greatly reduced. The surface of said nano- and microparticles may be provided with reactive chemical groups, in order to make possible covalent coupling of biomolecules or/and luminescent indicator dyes. Furthermore, the surface may be provided with chemical groups in order to prevent the particles from aggregating.

Luminescence measurement is a very common method in biological and chemical analysis. Its attractiveness is due to its high sensitivity, versatility and also the elimination of radiation exposure by radioactive

labeling reagents. In practice, luminescent markers distinguished by a high quantam yield are normally used. In most cases, the luminescence intensity of the luminescent marker is correlated with the sample parameter to be determined. Those determination methods are adversely affected by the fact that a multiplicity of factors interferes with the quantitative evaluation of luminescence intensity. Said factors may include firstly variations in the optical system (radiation intensity of the light source, detector sensitivity and transmission of the optical path), but also intrinsic optical properties of the sample (coloration or turbidity).

In order to eliminate or reduce said interfering influences, suitable methods for referencing the luminescence signals are required. WO 99/06821 (Klimant) describes a method for referencing luminescence signals, which is based on adding to the sample a luminescent reference dye which has similar (at best identical) spectral properties to the actual luminescent marker. In this way and in combination with frequence-modulated or time-resolved luminescence measurement, the intensity information is converted into a phase signal or a time-dependent parameter. In order to carry out correct referencing of the measurement signal in this way, inert luminescent reference standards are required, whose luminescence properties are not adversely affected by the sample parameters. Suitable for this purpose are, for example, phosphorescent inorganic solids such as, for example, Cr(III)-doped mixed oxides which can be admixed to the sample in powder form. On the other hand, it is also possible for this purpose to incorporate long-lived luminescent dyes into carriers made of organic or inorganic materials and admix the sample therewith.

Another type of interference of the quantitative evaluation of fluorescence intensity signals is the

occurrence of intrinsic fluorescence in the sample. Natural samples such as blood or serum, in particular, can have a multiplicity of fluorescent substances. If the signal intensity of the fluorimetric assay is very low, intrinsic fluorescence may even render the measurement impossible. A widespread method for removing the actual luminescence signal from the unspecific background signal is to use luminescent dyes with long-lived emission as markers. It is possible, with the aid of time-resolved luminescence techniques, to separate by time the delayed measurement signal from the short-lived background fluorescence. This method uses mainly phosphorescent chelates of the rare earth metals (in particular those of europium or terbium). However, said dyes have the disadvantage that they can only be excited by UV light sources. Moreover, the chelates are often unstable when used in soluble form in aqueous systems, i.e. the ligands are lost. However, suitable long-lived markers are potentially also luminescent metal/ligand complexes, in particular those with ruthenium(II) as central atom. If these dyes are added in soluble form to aqueous systems, their luminescence is normally quenched by molecular oxygen, strong oxidants or reducers.

Furthermore, it is also possible, for example for determining the pH, the concentration or activity of ions or small molecules, to use luminescent indicators whose luminescence intensity depends on the concentration or activity of the parameter to be determined, for example an analyte or the pH, due to direct or indirect interaction with the parameter to be determined, for example due to reaction with an analyte or as transducer.

All methods mentioned absolutely require the photophysical properties of the luminescent dye to be unaffected by the sample parameters. These preconditions are not met if such dyes are added in

dissolved form to the sample or contacted at least indirectly with the sample. Fluoresence or phosphoresence quenching by molecular oxygen and also oxidizing and reducing quenchers cause misinterpretations of the measurement signal.

In order to have available inert long-lived luminescent markers and luminescent dyes for referencing the luminescence intensity of luminescent indicators, the luminescent dyes have to be incorporated into solid materials so that they are incapable of interacting with the sample.

The present application describes both novel luminescent micro- and nanoparticles whose luminescence properties depend negligibly, if at all, on the sample composition, and methods for the preparation thereof. In addition, possible applications of the luminescent markers or luminescent dyes, present in the form of nano- and microparticles, for referencing the luminescence intensity of luminescent indicators are described.

The application therefore relates to luminescent, in particular phosphorescent, micro- and nanoparticles containing luminescent substances, for example metal/ligand complexes with long luminescence decay times, in a solid matrix so that they are shielded from ambient chemical parameters, for example a sample, and the luminescence properties of which, such as quantam yield, spectral characteristics, luminescence decay time or/and anisotropy, are essentially independent of the particular environment, for example the particular sample composition.

"Independent" in accordance with the subject application means that the dependence of the luminescence decay time and, where appropriate, further luminescence properties on the pO_2 and, where

appropriate, other interfering substances in the environment of the luminescent dyes which are present in the particles of the invention and are at least in indirect contact with the sample is lower then the dependence of the luminescence decay time and, where appropriate, further luminescence properties of the corresponding dyes which are at least in indirect contact with the sample, without the inventive shielding.

Preferably, the luminescence lifetime of the luminescent dyes present in the particles of the invention is in an air-saturated environment at most 20%, particularly preferably at most 15% and most preferably at most 10% shorter than in an O_2-free environment, in each case at room temperature. Without shielding, however, a reduction in the luminescence decay time by distinctly more than 80% is found in an air-saturated environment compared with an O_2-free environment.

The luminescent metal/ligand complexes are preferably compounds of transition metals such as ruthenium(II), osmium(II), rhenium(I), iridium(III), platinum(II) and palladium(II) as central atoms. The complex ligands are preferably selected from two- or/and three-dentate ligands with N-heterocycles, for example polypyridyl ligands such as 2,2'-bipyridine, bipyrazine, phenanthroline, terpyridil or derivatives thereof. Particularly preferred examples of metal/ligand complexes are the tris complexes of ruthenium(II) with 2,2'-bipyridyl, 1,10-phenanthroline, 4,4-diphenyl-2,2'-bipyridyl and 4,7-diphenyl-1,10-phenanthroline as ligands. Particular preference is furthermore given to carbonyl complexes of Re(I) with additional poly-N-heterocyclic ligands such as, for example, 2,2'-bipyridyl and 1,10-phenanthroline. Likewise, preferred metal/ligand complexes are the porphyrin complexes of Pt(II) or Pd(II) as central atom, which are distinguished by intense phosphorescence at room

temperature. The luminescence decay times of said compounds are preferably ≥ 100 nanoseconds, particularly preferably ≥ 400 nanoseconds. According to the invention, it is also possible to use rare earth metals such as, for example, the lanthanides Tb(III) and Eu(III) or other substances as long-lived luminescent dyes.

The average size of the luminescent micro- and nanoparticles is preferably in the range from 20 nm to 10 µm, particularly preferably from 50 nm to 1 µm. The luminescent compounds are incorporated into materials which are distinguished by low permeability (i.e. low diffusion constants and low solubility) for water, quenching gaseous substances (e.g. O_2) and interfering substances. Examples of suitable materials are nonporous glasses, in particular glasses which have been produced, for example, from silicon-, titanium-, zirconium- or tin-containing compounds, for example alcoholates such as tin tetraalcoholates, according to a sol/gel method.

Preparation of such glasses according to standard methods leads to materials which are characterized by a microporous structure. Incorporated luminescent dyes are thus accessible for dissolved sample components and in particular oxygen and can thus be quenched. For this reason, the sol glasses described in the present invention are, in a particular preparation step, compressed by heating to an elevated temperature of, for example, 200°C. After hydrolyzing the sol/gel precursor, for example tetramethoxysilane, the solvent is stripped off under reduced pressure and the sol/gel is dried prior to the final crosslinking. In this way, a dense nonporous glass matrix is formed. Biomolecules and also chemical compounds cannot penetrate said dense matrix and therefore do not influence the luminescence properties of the incorporated dyes. Inert phosphorescent sol/gel glasses having the dyes

ruthenium(II)-tris-1,10-phenanthroline and ruthenium(II)-tris-4,7-diphenyl-1,10-phenanthroline and a dye content of up to 40 mM (based on kg SiO_2) were produced according to said method. These materials are distinguished by intense luminescence at room temperature, which is not quenched by oxygen. Since the sol/gel phosphors are formed in the preparation process either in monolithic form or as thin films, microparticles have to be produced by powdering. Subsequent silanization of the particles leads to reactive surfaces which can be utilized for covalent coupling of luminescent indicators or biomolecules. For this, the particle surface may be provided with, for example, amino, epoxy, hydroxyl, thiol or/and carboxyl groups.

An alternative method of preparing inert luminescent particles is the use of organic polymers as embedding matrix, which are distinguished firstly by a very low gas permeability (in order to exclude oxygen) and secondly by minimum absorption of water (in order to prevent penetration of ionic compounds). Suitable polymers are polyvinyl chloride, polyvinylidene chloride, poly(meth)acrylic polymers and in particular polyacrylonitrile and also copolymers thereof.

Polyacrylonitrile (PAN) has an extremely low gas permeability, partly hydrophilic properties and a very low absorption capacity for water (approx. 2%). Moreover, the nitrile groups on the surface of the polymer particles, for example, can be saponified to give carboxyl groups or/and amide groups or converted to give amine groups, which are then available for covalent binding of various biomolecules. For this reason, polyacrylonitrile is the optimum embedding matrix for luminescent dyes as base for inert nano- and microparticles.

Furthermore, it is also possible to use polyacrylonitrile copolymers or mixed polymers with polyacrylonitrile, i.e. polymers containing acrylonitrile and additionally one or more monomers, in particular polyacrylonitrile copolymers or mixed polymers with at least 50%, preferably at least 70%, and particularly preferred at least 90%, by weight of PAN. A copolymer contains PAN and a comonomer in a polymer chain. A mixed polymer contains a PAN or PAN copolymer component in a polymer chain and at least one non-PAN component in another polymer chain. Suitable additional monomers for copolymers and mixed polymers are monomers with hydrophilic or/and reactive groups, for example acrylic acid, acrylic amines and acrylic esters, for example polyethylene glycol acrylic esters, or mixtures thereof. In this context, the hydrophilic groups are preferably concentrated on the particle surface. The hydrophilic or/and reactive groups on the surface can then be used for coupling binding partners such as biomolecules or luminescent indicator molecules. Furthermore, these groups can also contribute to preventing particle aggregation.

Luminescent micro- and nanoparticles based on polyacrylonitrile (PAN) can be prepared in various ways.

A. Precipitation of the particles from a solution of PAN or a PAN copolymer or mixed polymer in an organic solvent (mixture), for example dimethylformamide, by adding, dropwise in a controlled fashion, water, aqueous solutions, for example an NaCl solution, or other liquids which are miscible with the polymer solvent but cause a reduction in solubility and thus precipitation of the polymer with the luminescent dye. The polymer solution contains at the same time the dissolved luminescent dye. This method variant is particularly simple and therefore preferred.

B. Precipitation of the particles from a solution of PAN or a PAN copolymer or mixed polymer in an organic solvent (mixture), for example dimethylformamide, by adding, dropwise in a controlled fashion, water, aqueous solutions, for example an NaCl solution, or other liquids which are miscible with the polymer solvent, but cause precipitation of the polymer. The polymer solution contains no dissolved luminescent dye. The luminescent dye is introduced into the particles subsequently by diffusion.

C. Preparation of the particles by spraying a solution of PAN or a PAN copolymer or mixed polymer in an organic solvent (mixture), for example dimethylformamide, which contains the luminescent dye, for example, in water or ethanol, and evaporation of the solvent.

In all protocols it is possible to adjust the particle diameter specifically by altering the polymer proportion in the solution. With a decreasing proportion of polymer, the particle diameter is also reduced.

After preparing and isolating the luminescent micro- and nanoparticles, the surface can be activated by reactive carboxyl groups, for example by saponification of the surface-bound nitrile groups in base, for example concentrated sodium hydroxide solution. The carboxyl groups are required for two reasons. Firstly, it is possible to prepare in this way stable dispersions in (pH-)buffered systems and, secondly, biomolecules and luminescent indicators can be bound covalently to the surface.

Particles of the invention, whose surface has been modified by reactive groups, may be used for covalently coupling luminescent indicators or/and biomolecules. The luminescent indicators may be compounds similar to

those included in the particle matrix. In contrast to the included luminescent compounds, the luminescent indicators coupled to the surface are in contact with the environment, so that they can react to ambient chemical parameters. Particles modified in this way may be used as indicators with internal referencing. Alternatively, or additionally, it is also possible to couple biomolecules such as toxins, hormones, hormone receptors, peptides, proteins, lectins, oligonucleotides, nucleic acids, antibodies, antigens, viruses and bacteria to the particle surfaces. Coupling is carried out via known methods, for example by using bifunctional linker molecules.

In addition, it is possible to use the particles as standards for referencing luminescence intensity signals in fluorimetric assays, for example for diagnostic determination of analytes.

The micro- and nanoparticles may be used on the one hand as luminescent standards for converting the luminescence intensity of luminescent indicators bound to the surface or present in the environment into phase signals or time-dependent parameters (for example for referencing the luminescence intensity signal of optical luminescence sensors, with the particles being immobilized together with a luminescent indicator in a solid phase, as described in WO99/06821 (Klimant)),and on the other hand as luminescent markers for highly sensitive detection or determination of biomolecules.

The invention therefore also relates to a method for luminometric determination of a biochemical or chemical parameter using two different luminescent dyes which have different decay times and the time or phase characteristics of the resulting luminescent response are used for generating a reference parameter for determination of said parameter, with the first luminescent dye corresponding to said parameter at

least with respect to luminescence intensity and the second one essentially not corresponding to said parameter at least with respect to luminescence intensity and luminescence decay time and the method is characterized in that the second luminescent dye is used in the form of particles of the invention. The reference parameter used is preferably a ratio of the two luminescence intensity proportions, which is independent of the total intensity of the luminescence signal. A reference parameter which may be used as an alternative is the phase shift of the luminescence response of the first luminescent dye compared to that of the second luminescent dye. In addition, the reference parameter may also be the measured phase shift of the combined signal of the signal of the first luminescent dye and the delayed reference signal of the second luminescent dye. For further details of the method and a device for carrying out the method, WO99/06821 is referred to.

Furthermore, the following examples are intended to illustrate the invention.

Examples

Example 1

Preparation of luminescent nanoparticles from polyacrylonitrile and [ruthenium(II)-tris-4,7-diphenyl-1,10 phenanthroline]$^{2+}$

1 g of n-polyacrylonitrile (Polysciences Inc., MW 150000) is dissolved together with 10 mg of ruthenium(II)-tris-4,7-diphenyl-1,10-phenanthroline perchlorate in 100 ml of dimethylformamide (DMF) and introduced into a 1 l glass beaker. 400 ml of H_2O are slowly added dropwise to this solution with constant stirring, leading to a slight turbidity in the solution. This is followed by adding, likewise with constant stirring, 10 ml of a 5% strength sodium

chloride solution, resulting in a flocculent precipitate which settles at the bottom of the beaker overnight. This precipitate contains the entire dye and is separated by centrifugation and subsequently washed three times with 250 ml of a 0.5% strength NaCl solution. In the next step, the precipitate is washed with 200 ml of ethanol in order to wash out completely the luminescent dye adsorbed on the surface. The ethanol is removed from the precipitate by centrifugation. This is followed by a last washing step in a 0.05% strength NaCl solution. The precipitate which consists of the nanoparticles is removed and taken up in 50 ml of H_2O.

Example 2

Preparation of phosphorescent nanoparticles from polyacrylonitrile and [ruthenium(II)-tris-1,10 phenanthroline]$^{2+}$

1 g of n-polyacrylonitrile is dissolved together with 10 mg of ruthenium(II)-tris-1,10-phenanthroline hexafluorophosphate in 100 ml of dimethylformamide and introduced into a 1 l glass beaker. 400 ml of H_2O are slowly added dropwise to this solution with constant stirring, leading to a slight turbidity in the solution. This is followed by adding, likewise with constant stirring, 10 ml of a 5% strength sodium chloride solution, resulting in a precipitate which settles at the bottom of the beaker overnight. This precipitate contains approx. 90% of the dye used and is separated by centrifugation and subsequently washed three times with 250 ml of a 0.5% strength NaCl solution. In the next step, the precipitate is washed with 200 ml of ethanol in order to wash out completely the luminescent dye adsorbed on the surface. The ethanol is removed from the precipitate by centrifugation. This is followed by a last washing step in a 0.05% strength NaCl solution. The precipitate

(nanoparticles) is removed and taken up in 50 ml of H_2O.

Example 3

Carboxylation of the surface of the luminescent nanoparticles

10 ml of the particle suspension from Examples 1 or 2, having a solids content of 200 mg of polyacrylonitrile, are taken up in 50 ml of a 5% strength NaOH solution. The particles precipitate and the suspension is heated to 75°C with intense stirring for 45 minutes. An intense smell of ammonia indicates hydrolysis of the nitrile groups located on the surfaces. After clearing of the turbid solution, the sodium hydroxide solution is neutralized by adding HCl and adjusted to pH 3. This results again in precipitation of the particles carboxylated on the surface, which can then be removed by centrifugation. They are finally washed in 50 ml of buffer, pH 3, removed by centrifugation and taken up in 10 ml of distilled water.

The saponificationmay be carried out analogously also in 8% NaOH at 25°C for 24 h.

Example 4

Nanoparticles consisting of a copolymer of 90% polyacrylonitrile and 10% polyacrylic acid and [ruthenium(II)-tris-4,7-diphenyl-1,10-phenanthroline]$^{2+}$

2 g of a self-synthesized acrylonitrile/acrylic acid 10:1 copolymer and 40 mg of [ruthenium(II)-tris-4,7-diphenyl-1,10-phenanthroline]$^{2+}$ as trimethylsilylpropanesulphonate ($Ru(dphphen)_3TMS_2$) are dissolved in 400 g of DMF. 1 l of 10^{-3} N NaOH is added dropwise with stirring and water is added to 2 l. The clear suspension is adjusted to pH 3 with 0.1 N HCl and the precipitate is removed by centrifugation. The centrifugate is washed 3 times with in each case 1.8 l

of water and resuspended in 200 ml of 50 mM Na_2HPO_4 by means of ultrasound. The clear suspension is heated to approx. 80°C for 20 min and, after cooling, again adjusted to pH 3 by adding HCl, removed by centrifugation and resuspended in 200 ml of 50 mM Na_2HPO_4 by means of ultrasound.

Example 5

Nanoparticles comprising a copolymer of 95% polyacrylonitrile and 5% polyacrylic acid and $[Ru(dphphen)_3]^{2+}$

2 g of acrylonitrile/acrylic acid 20:1 copolymer and 40 mg of $Ru(dphphen)_3TMS_2$ are dissolved in 400 g of DMF. 1 l of 10^{-3} N NaOH is added dropwise with stirring and water is added to 2 l. The clear suspension is adjusted to pH 3 with 0.1 N HCl and the precipitate is removed by centrifugation. The centrifugate is washed 3 times with in each case 1.8 l of water and resuspended in 200 ml of 50 mM Na_2HPO_4 by means of ultrasound. The clear suspension is heated to approx. 80°C for 20 min and, after cooling, again adjusted to pH 3 by adding HCl, removed by centrifugation and resuspended in 200 ml of 50 mM Na_2HPO_4 by means of ultrasound.

Example 6

Nanoparticles consisting of a copolymer of 99.5% polyacrylonitrile and 0.5% polyacrylic amine and $[Ru(dphphen)_3]^{2+}$

0.5 g of acrylonitrile/3-aminopropylacrylamide - 200:1 copolymer and 10 mg of $Ru(dphphen)_3TMS_2$ are dissolved in 100 g of DMF. 0.5 l of 10^{-3} N HCl is added dropwise with stirring and water is added to 1 l. The clear suspension is adjusted to pH 9 with 0.1 N NaOH and the precipitate is removed by centrifugation. The centrifugate is washed 3 times with in each case 1 l of water and resuspended in 50 ml of water by means of ultrasound. The suspension is heated to approx. 80°C

for 20 min and, after cooling, washed 2 times with water and resuspended.

Example 7

Nanoparticles consisting of a copolymer of 90% polyacrylonitrile and 5% polyacrylic acid and 5% polyethylene glycol monoethyl ether acrylate and $[Ru(dphphen)_3]^{2+}$

0.5 g of acrylonitrile/acrylic acid/polyethylene glycol monomethyl ether acrylate 20:1:1 copolymer and 5 mg of $Ru(dphphen)_3TMS_2$ are dissolved in 200 g of DMF. 1 l of 10^{-3} N NaOH is added dropwise with stirring. The clear suspension is adjusted to pH 3 with 0.1 N HCl and the precipitate is removed by centrifugation. The centrifugate is washed 3 times with in each case 1 l of water and resuspended in 1 l of 100 mM Na_2HPO_4 by means of ultrasound. The clear suspension is adjusted to pH 3 by adding HCl, removed by centrifugation and resuspended in 200 ml of 100 mM Na_2HPO_4 by means of ultrasound. The clear suspension is heated to approx. 80°C for 20 min and, after cooling, again adjusted to pH 3 by adding HCl, removed by centrifugation and resuspended in 200 ml of 50 mM Na_2HPO_4 by means of ultrasound.

Example 8

Nanoparticles consisting of a copolymer of 85% polyacrylonitrile, 5% polyacrylic acid and 10% polysulfoacrylate and $[Ru(dphphen)_3]^{2+}$

0.5 g of acrylonitrile/acrylic acid/sulfopropylacrylate 20:1:2 copolymer and 50 mg of $Ru(dphphen)_3Cl_2$ are dissolved in 100 g of DMF. 0.5 l of 10^{-3} N NaOH is added dropwise with stirring. The clear suspension is adjusted to pH 3 with 0.1 N HCl and the precipitate is removed by centrifugation. The centrifugate is washed 3 times with in each case 1 l of water and resuspended in 100 ml of 50 mM Na_2HPO_4 by means of ultrasound. The

clear suspension is heated to approx. 80°C for 20 min and, after cooling, again adjusted to pH 3 by adding HCl, removed by centrifugation and resuspended in 100 ml of 50 mM Na_2HPO_4 by means of ultrasound.

Example 9

Characterization of luminescent particles based on polyacrylonitrile or polyacrylonitrile copolymers

The particles listed, having an average diameter of from 20 to 100 nm and containing the luminescent dye ruthenium(II)-tris-4,7-diphenyl-1,10-phenanthroline were measured in a 20 mM phosphate buffer (pH 7) at 20°C. The nanoparticles were dispersed in a sample. The results are shown in Table I below.

Table 1: Characterization of various phosphorescent nanoparticles based on polyacrylonitrile particles

(Diameter of the particles listed (20-100 nm), dye in all cases: the ruthenium(II)-tris-4,7-diphenyl-1,10-phenanthroline complex. All measurements were carried out in a 20 mM phosphate buffer (pH 7) at 20°C. The nanoparticles were dispersed in the sample.

Sensor	Base monomer (= acrylonitrile) [% (w/w)]	Comonomer(s)	Comonomer(s) [% (w/w)]	*Air-saturated* relative phosphorescence intensity I	decay time [µs]	*N_2-saturated* decay time [µs]	oxygen quenching (decrease in decay time between 0 and 200 hPa pO_2) in %
Dye dissolved in water	-	-	-	12	0.90	4.40	85
1 (Ex. 1)	100.0	-	0.0	23.81	5.69	6.20	8.2
2	90.0	acrylic acid	10.0	26.00	6.10	6.36	4.1
3	87.0	acrylic acid	13.0	19.81	5.55	6.17	10.0
4	76.9	acrylic acid	23.1	18.07	5.89	5.91	0.3

5 (Ex. 5)	95.0	acrylic acid	5.0	15.24	5.78	6.11	5.4
6	95.0	ethylene glycol monoethyl ether acrylate	5.0	19.36	6.01	6.24	3.7
7 (Ex. 7)	90.0	acrylic acid ethylene glycol monoethyl ether acrylate	5.0, 5.0	17.23	5.38	5.94	9.4
8	83.4	acrylic acid, ethylene glycol monoethyl ether acrylate	8.3, 8.3	19.46	6.00	6.16	2.6
9 (Ex. 8)	87.0	acrylic acid, acrylosulfonic acid	4.3, 8.7	16.05	5.36	5.98	10.4
10	95.0	primary acrylic amine (ester, $-CO(CH_2)_2NH_2$)	5.0	25.11	5.59	5.96	6.2
11	90.0	primary acrylic amine (ester, $-CO(CH_2)_2NH_2$)	10.0	18.64	5.75	5.82	1.2
12 (Ex. 6)	99.5	primary acrylic amine (amine, $-NH(CH_2)_3NH_2$)	0.5	16.52	5.27	5.90	10.7

Claims

1. A luminescent micro- or nanoparticle, characterized in that it contains luminescent substances having long luminescence decay times and said luminescent substances are essentially shielded from ambient chemical and biochemical parameters.

2. The particle as claimed in claim 1, characterized in that one or more luminescence properties of said luminescent substances, which are in particular selected from the group consisting of quantum yield, spectral characteristics, luminescence decay time and anisotropy, are essentially independent of the particular environment.

3. The particle as claimed in claim 1 or 2, characterized in that the luminescent substances are metal/ligand complexes of ruthenium(II), osmium(II) rhenium(I), iridium(III) platinum(II) and palladium(II) as central atom.

4. The particle as claimed in claim 3, characterized in that the luminescent substances are complexes with 2- or 3-dentate polypyridyl ligands such as 2,2'-bipyridine, bipyrazine, phenanthrolin, terpyridyl or derivatives thereof as ligands.

5. The particle as claimed in either of claims 3 - 4, characterized in that the luminescent compounds are the tris complexes of ruthenium(II) with 2,2'-bipyridyl, 1,10-phenanthroline, 4,4-diphenyl-2,2'-bipyridyl and 4,7-diphenyl-1,10-phenanthroline as ligands.

6. The particle as claimed in claim 1 or 2, characterized in that the luminescent substances are carbonyl complexes of Re(I) with additional diimine ligands such as derivatives of 2,2′-bipyridyl and 1,10-phenanthroline.

7. The particle as claimed in claim 1 or 2, characterized in that the luminescent compounds are porphyrin complexes of Pt(II) and Pd(II) as central atoms.

8. The particle as claimed in any of claims 1-7, characterized in that it contains an organic polymer which distinguishes itself by low absorption of water or/and minimum gas permeability.

9. The particle as claimed in claim 8, characterized in that it contains an organic polymer from the group consisting of polyacrylonitrile, poly(meth)acrylic copolymers, polyvinyl chlorides or polyvinylidene chlorides and copolymers thereof.

10. The particle as claimed in claim 9, characterized in that it contains polyacrylonitrile or polyacrylonitrile copolymers, in particular copolymers with acrylic acid, acrylic amines or/and acrylic esters.

11. The particle as claimed in any of claims 1-7, characterized in that it contains a glass which is essentially free of micropores.

12. The particle as claimed in claim 11, characterized in that

it contains a glass which has been produced according to a sol/gel process.

13. The particle as claimed in claim 11 or 12, characterized in that
it contains a sol/gel glass which has been prepared from silicon, titanium, zirconium or/and tin tetraalcoholates.

14. The particle as claimed in any of claims 1 - 13, characterized in that
its surface has been modified by reactive groups such as amino, epoxy, hydroxyl, thiol or/and carboxyl groups which make possible the covalent coupling of luminescent indicators or/and biomolecules.

15. The particle as claimed in claim 14, characterized in that
it contains luminescent indicators or/and biomolecules covalently coupled to its surface.

16. A method for preparing luminescent micro- and nanoparticles as claimed in any of claims 8 - 10, wherein the particles are precipitated from a polymer solution in which the luminescent compound is present in soluble form by adding a liquid dropwise, with the liquid being miscible with the polymer solvent but causing a reduction in the solubility of the polymer.

17. The method as claimed in claim 15 , wherein the particles are precipitated from a solution comprising dimethylformamide and polyacrylonitrile or polyacrylonitrile copolymer, in which the luminescent compound is present in soluble form, by adding water or an aqueous solution dropwise.

18. The method as claimed in claim 16 or 17, wherein the particle diameter is adjusted by varying the polymer content of the solution.

19. A method for preparing luminescent micro- and nanoparticles as claimed in any of claims 8-10, wherein the luminescent compound is incorporated by diffusion from a solvent (mixture) into already prefabricated particles.

20. A method for preparing luminescent micro- and nanoparticles as claimed in any of claims 8-10, wherein the particles are formed by spraying a polymer solution in which the luminescent compound is present in soluble form and evaporation of the solvent.

21. The method as claimed in claim 20, wherein the particle diameter is adjusted by varying the polymer content of the spray solution.

22. A method for preparing luminescent microparticles as claimed in any of claims 11-13, wherein the luminescent compound is incorporated into compressed monolithic sol/gel glasses which are subsequently ground and fractionated according to size.

23. The use of the luminescent micro- and nanoparticles as claimed in any of claims 1 - 14 for labeling and luminometric detection of biomolecules from the group consisting of toxins, hormones, hormone receptors, peptides, proteins, lectins, oligonucleotides, nucleic acids, antibodies, antigens, viruses and bacteria.

24. The use of the luminescent micro- and nanoparticles as claimed in any of claims 1 - 14

as reference standards of fluorescence intensity signals in fluorimetric assays.

25. The use as claimed in claim 23, wherein addition of the standard to the sample converts the intensity information into a phase signal or/and a time-dependent parameter.

26. The use of the luminescent micro- and nanoparticles as claimed in any of claims 1 - 14 for referencing the luminescence intensity signal of optical luminescence sensors, wherein the particles are immobilized to a solid phase together with a luminescent indicator.

27. A method for luminometric determination of a biochemical or chemical parameter using two different luminescent dyes which have different decay times and the time or phase characteristics of the resulting luminescent response are used for generating a reference parameter for determination of said parameter, with the first luminescent dye corresponding to said parameter at least with respect to luminescence intensity and the second one not corresponding to said parameter at least with respect to luminescence intensity and luminescence decay time,
characterized in that
the second luminescent dye is used in the form of particles as claimed in any of claims 1-15.

10/031506

 531 Rec'd PCT/F 15 JAN 2002

Claims

1. A luminescent micro- or nanoparticle, characterized in that it contains luminescent substances having long luminescence decay times and said luminescent substances are essentially shielded from ambient chemical, biochemical and gaseous parameters.

Claims

AMENDED CLAIMS

[filed at the International Bureau on February 24, 2001 (02.24.01); original claims 1-27 replaced by amended claims 1-27 (6 pages]

2. The particle as claimed in claim 1, characterized in that one or more luminescence properties of said luminescent substances, which are in particular selected from the group consisting of quantum yield, spectral characteristics, luminescence decay time and anisotropy, are essentially independent of the particular environment.

3. The particle as claimed in claim 1 or 2, characterized in that the luminescent substances are metal/ligand complexes of ruthenium(II), osmium(II) rhenium(I), iridium(III) platinum(II) and palladium(II) as central atom.

4. The particle as claimed in claim 3, characterized in that the luminescent substances are complexes with 2- or 3-dentate polypyridyl ligands such as 2,2'-bipyridine, bipyrazine, phenanthroline, terpyridyl or derivatives thereof as ligands.

5. The particle as claimed in either of claims 3 - 4, characterized in that the luminescent compounds are the tris complexes of ruthenium(II) with 2,2'-bipyridyl, 1,10-phenanthroline, 4,4-diphenyl-2,2'-bipyridyl and 4,7-diphenyl-1,10-phenanthroline as ligands.

6. The particle as claimed in claim 1 or 2, characterized in that the luminescent substances are carbonyl complexes of Re(I) with additional diimine ligands such as derivatives of 2,2'-bipyridyl and 1,10-phenanthroline.

7. The particle as claimed in claim 1 or 2, characterized in that the luminescent compounds are porphyrin complexes of Pt(II) and Pd(II) as central atoms.

8. The particle as claimed in any of claims 1-7, characterized in that it contains an organic polymer which distinguishes itself by low absorption of water or/and minimum gas permeability.

9. The particle as claimed in claim 8, characterized in that it contains an organic polymer from the group consisting of polyacrylonitrile, poly(meth)acrylic copolymers, polyvinyl chlorides or polyvinylidene chlorides and copolymers thereof.

10. The particle as claimed in claim 9, characterized in that it contains polyacrylonitrile or polyacrylonitrile copolymers, in particular copolymers with acrylic acid, acrylic amines or/and acrylic esters.

11. The particle as claimed in any of claims 1-7, characterized in that it contains a glass which is essentially free of micropores.

12. The particle as claimed in claim 11, characterized in that

it contains a glass which has been produced according to a sol/gel process.

13. The particle as claimed in claim 11 or 12, characterized in that it contains a sol/gel glass which has been prepared from silicon, titanium, zirconium or/and tin tetraalcoholates.

14. The particle as claimed in any of claims 1 - 13, characterized in that its surface has been modified by reactive groups such as amino, epoxy, hydroxyl, thiol or/and carboxyl groups which make possible the covalent coupling of luminescent indicators or/and biomolecules.

15. The particle as claimed in claim 14, characterized in that it contains luminescent indicators or/and biomolecules covalently coupled to its surface.

16. A method for preparing luminescent micro- and nanoparticles as claimed in any of claims 8 - 10, wherein the particles are precipitated from a polymer solution in which the luminescent compound is present in soluble form by adding a liquid dropwise, with the liquid being miscible with the polymer solvent but causing a reduction in the solubility of the polymer.

17. The method as claimed in claim 15, wherein the particles are precipitated from a solution comprising dimethylformamide and polyacrylonitrile or polyacrylonitrile copolymer, in which the luminescent compound is present in soluble form, by adding water or an aqueous solution dropwise.

18. The method as claimed in claim 16 or 17, wherein the particle diameter is adjusted by varying the polymer content of the solution.

19. A method for preparing luminescent micro- and nanoparticles as claimed in any of claims 8-10, wherein the luminescent compound is incorporated by diffusion from a solvent (mixture) into already prefabricated particles.

20. A method for preparing luminescent micro- and nanoparticles as claimed in any of claims 8-10, wherein the particles are formed by spraying a polymer solution in which the luminescent compound is present in soluble form and evaporation of the solvent.

21. The method as claimed in claim 20, wherein the particle diameter is adjusted by varying the polymer content of the spray solution.

22. A method for preparing luminescent microparticles as claimed in any of claims 11-13, wherein the luminescent compound is incorporated into compressed monolithic sol/gel glasses which are subsequently ground and fractionated according to size.

23. The use of the luminescent micro- and nanoparticles as claimed in any of claims 1 - 14 for labeling and luminometric detection of biomolecules from the group consisting of toxins, hormones, hormone receptors, peptides, proteins, lectins, oligonucleotides, nucleic acids, antibodies, antigens, viruses and bacteria.

24. The use of the luminescent micro- and nanoparticles as claimed in any of claims 1 - 14

as reference standards of fluorescence intensity signals in fluorimetric assays.

25. The use as claimed in claim 23, wherein addition of the standard to the sample converts the intensity information into a phase signal or/and a time-dependent parameter.

26. The use of the luminescent micro- and nanoparticles as claimed in any of claims 1 - 14 for referencing the luminescence intensity signal of optical luminescence sensors, wherein the particles are immobilized to a solid phase together with a luminescent indicator.

27. A method for luminometric determination of a biochemical or chemical parameter using two different luminescent dyes which have different decay times and the time or phase characteristics of the resulting luminescent response are used for generating a reference parameter for determination of said parameter, with the first luminescent dye corresponding to said parameter at least with respect to luminescence intensity and the second one not corresponding to said parameter at least with respect to luminescence intensity and luminescence decay time
characterized in that
the second luminescent dye is used in the form of particles as claimed in any of claims 1-15.

22491P US-WO

(Foreign associate use only)

DECLARATION AND POWER OF ATTORNEY

Attorney's Docket No. **18744-0004**

As a below named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name. I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: **Production and Use of Luminescent Microparticles and Nanoparticles**, the specification of which

☐ is attached hereto.

☐ was filed on ______ as PCT International Application No. ______ and was amended (if applicable) on ______.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above. I do not know and do not believe that the same was ever known or used by others in the United States of America before my or our invention thereof, or patented or described in any printed publication in any country before my or our invention thereof or more than one year prior to the date of this application. I further state that the invention was not in public use or on sale in the United States of America more than one year prior to the date of this application. *I understand that I have a duty of candor and good faith toward the Patent and Trademark Office*, and I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below any foreign application for patent or inventor's certificate disclosing subject matter in common with the above-identified specification and having a filing date before that of the application on which priority is claimed:

Country	App. No.	Date of Filing	Priority Claimed Under 35 USC §119
Germany	199331049	July 15, 1999	Yes ✓ No ____

I hereby claim the benefit under Title 35, United States Code, § 120 of any prior United States application(s), or §365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each claim of the present application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations §1.56, which became available between the filing date of the prior application and the national or PCT international filing date of this application:

Application No.	Filing Date	Status: patented, pending, abandoned
PCT/EP00/06832	July 17, 2000	Pending

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statement were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patents issuing thereon.

I hereby authorize the U.S. attorneys named herein to accept and follow instructions from **Weickmann & Weickmann**, as to any action to be taken in the Patent and Trademark Office regarding this application, without direct communication between the U.S. attorney and the undersigned. In the event of a change in the persons from whom instructions may be taken, the U.S. attorney named herein will be notified by the undersigned.

9

POWER OF ATTORNEY: The following attorneys are hereby appointed to prosecute this application and transact all business in the Patent and Trademark Office connected therewith: Peter G. Pappas ~~- 33,205~~; Daniel J. Warren - 34,272; William L. Warren - 36,714; Michael S. Pavento - 42,985; Lisa M. Cobern ~~- 44,669~~; Robert A. Lester - 24,004; Erinn C. Kelly - 44,822; Jerry C. Liu - P47,754; Kevin W. King - 42,737.

Send correspondence to: **SUTHERLAND ASBILL & BRENNAN LLP**
999 Peachtree Street, NE
Atlanta, GA 30309-3996

Direct telephone calls at (404) 853-8000 to
William L. Warren

IW

Full name of sole or first inventor:	Ingo Klimant
Citizenship:	Germany
Residence:	~~Friedrich-Ebert-Strasse 32, D-93051 Regensburg,~~ GERMANY
Post Office Address:	
Inventor's signature	X Ingo Klimant — Date: X 5.1.02

AO 657693.1

ACKERWEG 4, D-93098 MINTRACHING, GERMANY